Method Development and Optimization for the Preparation and Analysis of Starch Hydrolysis Products using NMR Spectroscopy with Beer as a Surrogate Matrix

Research Thesis

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Contents

Abstract……..……..2

Acknowledgements……..3

List of Abbreviations and symbols…….…...……...……...…….……..4

Chapter1: Introduction..…….……...…...…5

Starch……...5

Starch hydrolysis……....…...…….……...……...5-6

Beer processing…….……...6

Mashing…….…….…...7-8

Top versus Bottom fermentation……..…….……...8

NMR Spectroscopy in food/beverage analysis……...……...…….…….9-11

Major research goals…….…….……..……...….…..10

Chapter2: Current Literature……....…….……...…….12

Chapter 3: Materials and Methods…….……...……...….…….14

A. Beer samples for NMR analysis……..……....……...…14

B. Partial hydrolysis of wheat starch (heat denaturation)……..…....……15-16

C. Partial hydrolysis of wheat starch (alcohol denaturation).....16-17

D. 1H NMR Analysis……..17

Chapter 4: Results and Discussion……..……....18

Chapter 5: Conclusions and Recommendations…….…...23-24

Abstract

A basic benchtop method was tested for the emulation of enzymatic starch hydrolysis, the principle process that occurs in brewing beer that determines the production of fermentable and non-fermentable sugars. Several signals belonging to hydrolysis products appear in the 1H NMR spectrum, indicating that NMR has the potential to become an efficient tool for quantification and kinetics studies for starch hydrolysis. In addition, various beer samples were analyzed using 1H NMR spectroscopy with the purpose of discrimination of beer varieties under the classification of top and bottom fermentation. Special attention was given to the spectral regions of δ 3.4-4.0 and δ 4.5-5.4 ppm, which correspond with the carbohydrate composition of the substrate. A modified pulse sequence based on the standard 1D noesy experiment with presaturation and shaped pulses was used to achieve the simultaneous suppression of the three signals belong to water and ethanol. Although compositional variations were reflected on the 1H NMR spectra obtained by the analysis of different samples of beer, as of yet we have been unable to differentiate beers based upon the class identity of top or bottom fermenting yeast utilization. These results indicate that other factors may also play an important role on beer composition and/or a larger set of samples is required before drawing final conclusions.

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List of Abbreviations and Symbols

ADC	Apparent diffusion coefficient
ANOVA	Analysis of variance
LDA	Linear discriminant analysis
MVSA	Multivariate statistical analysis
NMR	Nuclear magnetic spectroscopy
NOESY	Nuclear overhauser effect spectroscopy
PBS	Phosphate buffered saline
PCA	Principal component analysis
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
WET	Water suppression enhanced through T1
δ	Chemical shift scale expressed in ppm

Chapter 1: Introduction

Starch

Starch is the primary energy storage material in plants and it is extensively used in the food industry for food texture modification, the production of syrups, and other applications.[1] In addition, it is a vital precursor for the development of fermentable and non-fermentable sugars in beer. The structure of starch consists of two major components: amylose and amylopectin. Amylose is long and non-branching and composed of alpha-D-glucose units, while amylopectin is a highly branched structure consisting of glucosyl units. Amylopectin typically occurs in a much higher degree than amylose, although mutant varieties of starch can vary and even have a dominance of amylose. Similarly, there are varieties of starch that are nearly entirely composed of amylopectin, those of which are called "waxy starch". [2]

Starch Hydrolysis

The process of starch hydrolysis enables the production of numerous functional hydrolysis products, such as glucose syrups and maltodextrins. Amylases, the enzymes that perform the hydrolysis of starch and are known in the context of beer as diastatic enzymes[3], compose 25% of the industrial enzyme market chiefly due to their potential to modified and reduce starch for use in not only the food industry, but extending also to pharmaceuticals, consumer products, textile, and chemical manufacturing.[4] The starch hydrolysis process consists of multiple parts, most

[1]Maarel, Marc J.e.c Van Der, Bart Van Der Veen, Joost C.m Uitdehaag, Hans Leemhuis, and L. Dijkhuizen. "Properties and Applications of Starch-converting Enzymes of the α-amylase Family." Journal of Biotechnology 94, no. 2 (2002): 137-55. doi:10.1016/s0168-1656(01)00407-2.

[2] Evans, D. E., C. Li, and J. K. Eglinton. "The Properties and Genetics of Barley Malt Starch Degrading Enzymes." Advanced Topics in Science and Technology in China Genetics and Improvement of Barley Malt Quality, 2009, 143-89. doi:10.1007/978-3-642-01279-2_6.

[3] Evans, 78

[4] Souza, Paula Monteiro De, and Pérola De Oliveira E Magalhães. "Application of Microbial α-amylase in Industry - A Review." Brazilian Journal of Microbiology 41, no. 4 (2010): 850-61. doi:10.1590/s1517-83822010000400004.

pertinent being liquefaction, saccharification, and isomerization. Prior to any of these steps, a separate process referred to as gelatinization must occur for the development of starch products in high quantity at a reasonable speed. For gelatinization to occur, the starch suspension must be brought to a certain temperature, which varies depending upon the source starch. Under these conditions, the ordered structure of starch becomes hydrated and swells, which disrupts the ordered structure that exists in native starch. It is this transformation which allows for access of amylolytic enzymes to the substrate and thus highly enhances the speed of starch hydrolysis. Following gelatinization, the gelatinized starch product is accessible for partial hydrolysis by *alpha-amylase.* It is important to note that this process depends on the presence of calcium ion (Ca^{2+}) as a co-factor.[5] It is *beta-amylase* that then performs further hydrolysis on the substrate, acting on the non-reducing ends of amylose and amylopectin. This then enables the reduction of starch into its fermentable constituents. Importantly, the thermostability of *beta-amylase* is much less than *alpha-amylase* and it begins to denature at temperatures higher than 55 °C. This is an important aspect of its role in beer carbohydrate product, as this vulnerability to denaturation limits the amount of fermentable sugars that can be produced by the endogenous enzymes.[6]

Beer processing

The elaborate nature of the process of brewing leads to the immense complexity of beer, which makes it an ideal surrogate matrix for the study of a number core constituents, including organic acids, carbohydrates, amino acids, and other components. To achieve the finished product that is known as beer, there is a general process that is modified to produce varying results and the

[5] Zhang, Guoping, and Chengdao Li. Genetics and Improvement of Barley Malt Quality. Berlin, Heidelberg: Springer Berlin Heidelberg, 2010.
[6] Evans, 79

different varieties of beer that are on the market today. The process begins with malting, which involves the drying of grains, chiefly cereal grains, then subsequent steeping of grains to facilitate germination. This product is then dried and heated in a kiln to various roast levels. Once this "malt" is generated, it is transferred to a "mash tun", within which the malt is heated. This process occurs at approximately 68 °C in a single infusion mash and liberates the various amylase enzymes inherent within the malted grains so that they may begin to hydrolyze the starches within the grain into fermentable and non-fermentable sugars. The product of mashing is transferred to a lautering tun, within which the sugary liquid content of the mash (the "wort") is separated from the spent grain. In the primary brewing process that is the most well-known, the wort is combined with hops in a kettle and boiled, which contribute much of the characteristic flavor of beer. However, it is worthy of note that the hops impart very little in terms of sugar content. The product of the kettle is cooled to conditions sufficient for the sustainability of yeast activity and separated from the spent hops. Finally, yeasts are added to begin fermentation, with the two primary varieties of yeasts utilized being *Saccharomyces cerevisiae* (top fermenting yeast) and *Saccharomyces pastorianus* (bottom fermenting yeast). It is within the fermentation process that the small-sized, fermentable sugars that result from mashing are consumed by the added yeast and alcohol and carbon dioxide are produced. [7]

Mashing

The process that produces the wort, as mentioned above, is referred to as mashing. The enzymes liberated from the grist in the mashing process include *alpha-amylase, beta-amylase, cytaste, peptidase, proteinase, limit dextrinase, phytase,* and *beta-glucanase.* The parameters and

[7] Briggs, D. E., P. A. Brookes, R. Stevens, and C. A. Boulton. Brewing, Science and Practice: Science and Practice. Cambridge: Woodhead Pub., 2004.

the methodology of the mash can be modified to increase or decrease the degree of activity for each respective enzyme. The principle enzymes for the hydrolysis, or amylolysis, of starch are *alpha* and *beta-amylase*.[8] The role of *alpha-amylase* is the digestion the 1-4 alpha bonds of starch into smaller, yet often non-fermentable maltodextrins, which are highly branched polysaccharides that result from the amylolysis of amylopectin, in a process called *liquefaction.* The role of *beta-amylase* in amylolysis is the subsequent attack of amylose chains, removing the maltose units. The optimum temperature and pH range at which *alpha-amylase* is active, is a pH of 5.0-5.7 and a temperature of 72-75 °C.[9]

The brewing industry has long recognized the impact of enzyme concentration and the utility of enzyme modification in the process of producing wort. Enzymes can also be added exogenously, or external to the inherent addition of the malting grain. A popular exogenous enzyme used in the brewing process as an adjunct is amyloglucosidase, a glucogenic exoamylase which converts dextrins into D-glucose through breakage of the a-1,4 linkages at non-reducing terminals.[10] This results in the total hydrolysis of starch, which is otherwise achieved through the tandem activity of *alpha* and *beta-amylase*.

Top vs bottom fermented beers

Beers exist in many varieties, although among these varieties typically exists a broad categorization into two discrete groups: top and bottom fermented beers. This classification refers to the behavior of the strains utilized in each case. Top-fermentation involves the yeast

[8] Sammartino, Mark. Enzymes in Brewing 52, no. 3 (2015): 156-64. MBAA TQ.
[9] Sammartino, 159
[10] Espinosa-Ramírez, Johanan, Esther Pérez-Carrillo, and Sergio O. Serna-Saldívar. "Maltose and glucose utilization during fermentation of barley and sorghum lager beers as affected by alpha-amylase or amyloglucosidase addition." Journal of Cereal S

Saccharomyces cerevisiae and is associated with ale-type beers. Such beers are called "top-fermenting" due to the propensity of this strain of yeast to naturally float to the top of the fermentation tun. Likewise, bottom-fermentation involves the yeast *Saccharomyces pastorianus* and is associated with the production of lager-type beers, and typical behavior involves the yeast sinking to the bottom in fermentation.[11]

NMR Spectroscopy and its role in food/beverage analysis

NMR Spectroscopy is an analytical technique which utilizes the magnetic properties of specific nuclei that have a non-zero nuclear spin, predominantly 1H and 13C but also 31P, 19F, and 15N. Nuclei with nuclear spin number of ½ generally possess convenient properties for NMR and thus produce NMR spectra of high quality. Nuclei, such as 1H and 31P have high natural abundance and they provide a sensitive analysis, whereas nuclei such as 13C and 15N are of low natural abundance and they require longer experimental times and are much less sensitive than the former techniques. Among other techniques commonly utilized within food science and chemistry as a whole, NMR is commonly used due to its non-destructive nature, speed, and robust availability of structural information, although there are various setbacks prohibiting its wider utilization within the food sciences including high cost and a perceived "barrier to entry" due to its technological complexity. Despite this, it is a technique that has begun to gain momentum within food science. The major principle behind NMR analysis is that magnetization of a sample that contains NMR active nuclei and is located inside a strong field NMR magnet is excited using radio-frequency pulses and during its relaxation back to equilibrium a signal is generated. NMR can be an excellent tool for compositional analysis and reaction monitoring and when combined

[11] Pires, Eduardo, and Tomáš Brányik. Biochemistry of Beer Fermentation. Cham: Springer International Publishing, 2015.

with statistical analysis it can provide valuable information for a holistic product assessment. NMR can be combined with univariate statistical analysis techniques such as t-tests and ANOVA, but also with multivariate statistical analysis (MVSA) methods. MVSA include supervised approaches where the class membership of the samples is known and unsupervised techniques where the class membership of each sample is not known. Typical examples for supervised and unsupervised MVSA methods include principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA).[12]

Major Research Goals

Beer is an ideal surrogate matrix for the analysis of carbohydrates due to its vast array of carbohydrates contributing to flavor, aroma, and body, as well as the many possible modifications that exists between the initial substrate and the finished product. There is also an idyllic system where enzymatic hydrolysis naturally occurs and degrades starch into smaller sugars. Very often in analytical chemistry substrates are modified through acid or base hydrolysis in order to obtain a byproduct that can be easily studied using analytical techniques. In particular, starch hydrolysis can be efficiently performed via either acid or enzymatic means. However, enzymatic hydrolysis has many advantages over acid hydrolysis: particularly, there is no generation of corrosive waste, which could classify it as a greener technique. It also possesses a lower potential for the formation of unwanted or harmful byproducts.[13]

[12] Hatzakis, Emmanuel. "Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science: A Comprehensive Review." Comprehensive Reviews in Food Science and Food Safety 18, no. 1 (2018): 189-220. doi:10.1111/1541-4337.12408.
[13] Wahlstroem, R. M., and A. Suurnaekki. "Enzymatic Hydrolysis of Lignocellulosic Polysaccharides in the Presence of Ionic Liquids." Green Chemistry 46, no. 2 (2015): 694-714. doi:10.1002/chin.201516336.

The question was if the non-fermentable sugars present in beer, which are a product of the hydrolysis that occurs in the mash, can be used for the discrimination of different types of beer, particularly relating to the categorizations based upon the yeast type (top versus bottom fermenting yeasts, or *Saccharomyces cerevisiae* versus *Saccharomyces pastorianus)*. Previous studies have shown a correspondence between the carbohydrate composition of the finished product and the strain of yeast utilized in fermentation.[14] This correspondence is logical-- different yeasts will utilize/consume the wort differently, resulting in different products post-fermentation. However, we hypothesized that through analysis of the carbohydrate composition of beers of broad sub-categorization, yet with an even split among top versus bottom fermented varieties, it would be possible to distinguish these beers through multivariate analysis by their yeast categorization alone. However, this analysis is not without caveats. Beers possess an immense amount of variety and much of the protocols behind the processing of different beers remain undisclosed or is not immediately available information.

Another major facet of the project was to develop various method optimizations for the analysis of beer using NMR spectroscopy, focusing both on methods of sample preparation but also including various optimization methodologies in the acquisition of NMR spectra. One particular obstacle in the analysis of beverage matrices is the predominance of a water peak and the two strong signals of ethanol that cannot be suppressed effectively by the available pulse sequences such as presaturation, excitation sculpting, and WET.

Lastly, methods for benchtop starch hydrolysis were developed incorporating parameters common to those seen in a beer mash. Two different methods were tested to ensure the deactivation

[14] Stewart, Graham. "Saccharomyces Species in the Production of Beer." Beverages 2, no. 4 (2016): 34. doi:10.3390/beverages2040034.

of the amylolytic enzymes: one through heat denaturation and the other through the use of an alcohol.

Chapter 2: Current literature

Beer has been previously evaluated using NMR for purposes of quality control and characterization based upon geography. Much of this work focuses on the analysis of organic acids in beer, which play an important role in the various qualities of the final product. A study by Nord et al. utilized 1H NMR to quantify organic and amino acids in 58 samples of lager-type beers produced by 20 different brands. Spectra were obtained using a 600 MHz instrument and possessed D2O as a lock solvent. The δ 1.4-3.2 ppm range consisted of weak signals originating from organic acids of interest, while the amino acids were in the δ 6.5-8.8 ppm range.[15]

While literature currently exists on the analysis of beer using NMR spectroscopy, these analyses have been focused on carbohydrates to a limited degree. However, there are notable exceptions. A paper by Petersen et al. utilized 1H NMR for the profiling of carbohydrates in beer and it is from this paper that much of the sample preparation protocol was developed, although numerous modifications to this method were tested.[16] In addition, in a recent study authors used NMR combined with unsupervised and supervised analysis for the successful discrimination of Brazilian lager beers based upon the utilization of adjuncts, or alternative carbohydrate sources often employed by macrobreweries to cut down on overall cost. Similar to our study, their interest was in the carbohydrate variations that occurred among beers within spectra and they utilized these

[15] Nord, Lars I., Pia Vaag, and Jens Ø. Duus. "Quantification of Organic and Amino Acids in Beer By 1H NMR Spectroscopy." Analytical Chemistry 76, no. 16 (2004): 4790-798. doi:10.1021/ac0496852.

[16] Petersen, Bent O., Mathias Nilsson, Marie Bøjstrup, Ole Hindsgaul, and Sebastian Meier. " 1 H NMR spectroscopy for profiling complex carbohydrate mixtures in non-fractionated beer." Food Chemistry 150 (2014): 65-72. Accessed April 3, 2019. doi:10.1016/J.FOODCHEM.2013.10.136.

differences as a means for discriminant analysis.[17] In another recent study authors combined NMR with MVSA and they were able to discriminate between lager and ale type beers, although they had issues associated with water and ethanol resonances and dealt with these through water suppression and lyophization to deal with the ethanol[18]. A study conducted by Lachenmeier et al. exists on the analysis of beer using 1H NMR spectroscopy which also applies water-ethanol suppression techniques, although these analyses were conducted using a 400 MHz spectrometer.[19] The work performed in this study focuses on a similar NMR technique for the sake of further validation of the inclusion of NMR in carbohydrate analytical methods.

A large number of studies currently exist concerning the development of various starch hydrolysis methods. Since the focus of this study was on residual sugars that exist following fermentation, key hydrolysis studies focused on the products generated predominantly through partial hydrolysis with *alpha-amylase.* In particular, the hydrolysis experiments conducted here drew inspiration from a study by Rodríguez et al., in which starch was partially hydrolyzed with an *Bacillus licheniformis* sourced *alpha-amylase* solution (Termamyl 300 L Type DX) at a steady pH of 7.5 with substrate concentrations between 0.25 to 2.00 g/L and enzyme (solution) concentrations ranging from $0.575 \times 10-4$ and $13.8 \times 10-4$ g/L tested.[20]

[17] Silva, Luis Augusto Da, Danilo Luiz Flumignan, Aristeu Gomes Tininis, Helena Redigolo Pezza, and Leonardo Pezza. "Discrimination of Brazilian Lager Beer by 1H NMR Spectroscopy Combined with Chemometrics." Food Chemistry 272 (2019): 488-93. doi:10.1016/j.foodchem.2018.08.077.
[18] Jeong, Ji-Ho, Sung-Jin Cho, and Yongae Kim. "High-Resolution NMR Spectroscopy for the Classification of Beer." Bulletin of the Korean Chemical Society 38, no. 4 (2017): 466-70. doi:10.1002/bkcs.11113.
[19] Lachenmeier D. W.; Frank W.; Humpfer E.; Schäfer H.; Keller S.; Mörtter M.; Spraul M. Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis. Eur. Food Res. Technol. 2005, 220 (2), 215–221.
[20] Bravo Rodriguez, V., E. Jurado Alameda, J.f. Martinez Gallegos, A. Reyes Requena, and A.i. Garcia Lopez."Enzymatic Hydrolysis of Soluble Starch with an α-Amylase from Bacillus Licheniformis." Biotechnology Progress 22, no. 3 (2006): 718-22. doi:10.1021/bp060057a.

Studies have been conducted observing the products of starch hydrolysis using solution-state NMR, although to our knowledge no such study has drawn a comparison between these products as observed with NMR and the finished products of beer.

Chapter 3: Materials and Methods

Chemicals

Wheat starch and *alpha-amylase* enzyme were purchased from Sigma Aldrich. Wheat starch was used in this analysis as a satisfactory substitute for barley starch, as the latter is difficult to obtain in small commercial quantities. *Alpha-amylase* enzyme was stored at freezing when not in use. D_2O, CD_3OD and TSP (used as internal reference in later tests) were purchased Cambridge Isotope Laboratories (Tewksbury, MA).

Sample preparation

Protocols

A. *Beer samples for 1H NMR analysis**

a. Transfer 3 mL of sample from container of purchase into a 5 mL glass vial.
b. Shake/agitate the sample for 5 minutes, degassing by removing cap every 30 seconds
c. Transfer 570 uL of sample into a 5.0 mm NMR tube. Add 30 uL of deuterated water to the vial. Optionally, add small quantity of TSP as internal reference. Store in freezer until further analysis.

*Sample preparation method was adapted from a paper by B. O Petersen. [21]

[21] Petersen, Bent O., Mathias Nilsson, Marie Bøjstrup, Ole Hindsgaul, and Sebastian Meier. " 1 H NMR spectroscopy for profiling complex carbohydrate mixtures in non-fractionated beer." Food Chemistry 150 (2014): 65-72. Accessed April 3, 2019. doi:10.1016/J.FOODCHEM.2013.10.136.

A number of different NMR methodologies were tested for the analysis of beer samples. Predominant goals were to minimize the presence of water and ethanol peaks. Additionally, the use of deuterated buffer was also tested so as to maintain the pH of the sample for analysis, although this method was not carried into the final analysis.

As a preliminary study, three varieties of beer were purchased from Lucky's Market in Columbus, Ohio that were brewed with different kinds of yeast (*Saccharomyces cervisiae*, or ale, *Saccharomyces pastorianus*, or lager), and a particular variety of *cervisiae* yeast known as Norwegian Voss Kveik (milk stout).[22]

Further beer analysis involved the purchase of 23 distinct beers from Lucky's Market in Columbus, Ohio, which were randomized and prepared following the above protocol.

B. Partial hydrolysis of wheat starch (with heat denaturation)

1. Preparation of PBS buffer

- In 800 mL of DI water
    - 8 g NaCl
    - 0.2 g KCl
    - 1.44 g Na_2HPO_4
    - 0.24 g KH_2PO_4
    - 0.133 g $CaCl_2 \cdot 2H_2O$
- Adjust the pH of buffer to 5.5 using 1 M hydrochloric acid
- Dilute solution to 1 L

[22] Preiss, Richard, Caroline Tyrawa, Kristoffer Krogerus, Lars Marius Garshol, and George Van Der Merwe. "Traditional Norwegian Kveik Are a Genetically Distinct Group of Domesticated Saccharomyces Cerevisiae Brewing Yeasts." Frontiers in Microbiology 9 (2018). doi:10.3389/fmicb.2018.02137.

2. Dissolve 0.2 g of wheat starch in 100 mL of PBS buffer. Stir wheat starch until it is fully dissolved and the solution opaque.

3. Heat the starch solution to 68 °C. Gelatinization should begin at approximately 64 °C and conclude at 68 °C. The solution should appear to be near-transparent.

4. Place solution in 68 °C hot water bath and add 3 mL of *alpha-amylase* solution. Allow solution to incubate for one hour to ensure completion of partial hydrolysis.

5. Remove 570 uL of solution and transfer to a glass test tube. Submerge the test tube in boiling water for 1 minute to ensure the denaturation of the enzyme.

6. Transfer solution to a 5 mm NMR tube and add 30 uL of deuterated methanol (CD3OD). Store samples in freezer until further analysis.

C. Partial hydrolysis of wheat starch (with alcohol denaturation)

1. Preparation of PBS buffer

- In 800 mL of DI water
    - 8 g NaCl
    - 0.2 g KCl
    - 1.44 g Na_2HPO_4
    - 0.24 g KH_2PO_4
    - 0.133 g $CaCl_2 \cdot 2H_2O$
- Adjust the pH of buffer to 5.5 using 1 M hydrochloric acid
- Dilute solution to 1 L

2. Dissolve 0.2 g of wheat starch in 100 mL of PBS buffer. Stir wheat starch until it is fully dissolved and the solution opaque.

3. Heat the starch solution to 68 °C. Gelatinization should begin at approximately 64 °C and conclude at 68 °C[23]. The solution should appear to be near-transparent.

4. Place solution in 68 °C hot water bath and add 3 mL of *alpha-amylase* solution. Allow solution to incubate for one hour to ensure completion of partial hydrolysis.

5. Remove 570 uL of solution and transfer to a glass test tube. *Immediately* add 30 uL of deuterated methanol to ensure denaturation proceeds as soon as possible.

6. Transfer solution to a 5 mm NMR tube. Store samples in freezer until further analysis.

D. *1H NMR Analysis*

Beer samples were analyzed on both the 700 MHz instrument and the 800 MHz instruments. For 1H NMR analysis, water suppression was a pertinent technique for the successful elucidation of spectra within the complex carbohydrate region since there is overlap within this region between key carbohydrate spectra and water, a peak for which is very intense due to the high concentration of water in both beer and benchtop hydrolyzed samples. Two different techniques were tested: excitation sculpting, an efficient methodology used for the suppression of the water peak via 180° water-selective pulses, and 1D NOESY with presaturation, a more commonly applied technique.

Data analysis

Data spectral processing was performed on each of the 23 beer samples for uniform baseline and phase correction in Topspin 3.2 (details of suite). For chemometric analysis the spectral regions δ 0.50−10.00 was integrated into regions with equal width of 0.05 ppm using the

[23] Yuryev, Vladimir P., Attilio Cesàro, and Wolfgang J. Bergthaller. Starch and Starch Containing Origins: Structure, Properties, and New Technologies. New York: Nova Science Publishers, 2002.

AMIX software package (V3.9, Bruker-Biospin) and MVSA was carried out with SIMCA-P+ software (version 14.1, Umetrics, Sweden). Data were mean-centered and scaled using Pareto method. Log-transformation was also applied to achieve an improved normal distribution of the data.

Chapter 4: Results and Discussion

1H NMR analysis of beer samples

1H NMR analysis is a promising tool for the compositional analysis of beer as many important metabolites that can be related to beer quality can be identified. 1H NMR analysis is also able to detect variations in the metabolite composition of various types of beer, as found by our preliminary studies. As shown in **Figure 1**, despite the spectral similarities between three types of beer, namely a lager, Belgian pale and milk stout prepared using Voss Kveik yeast, specific compounds appear in different levels in each beer and in some cases new molecules appear in the spectrum. This preliminary data led us to perform a larger study where 23 samples were examined.

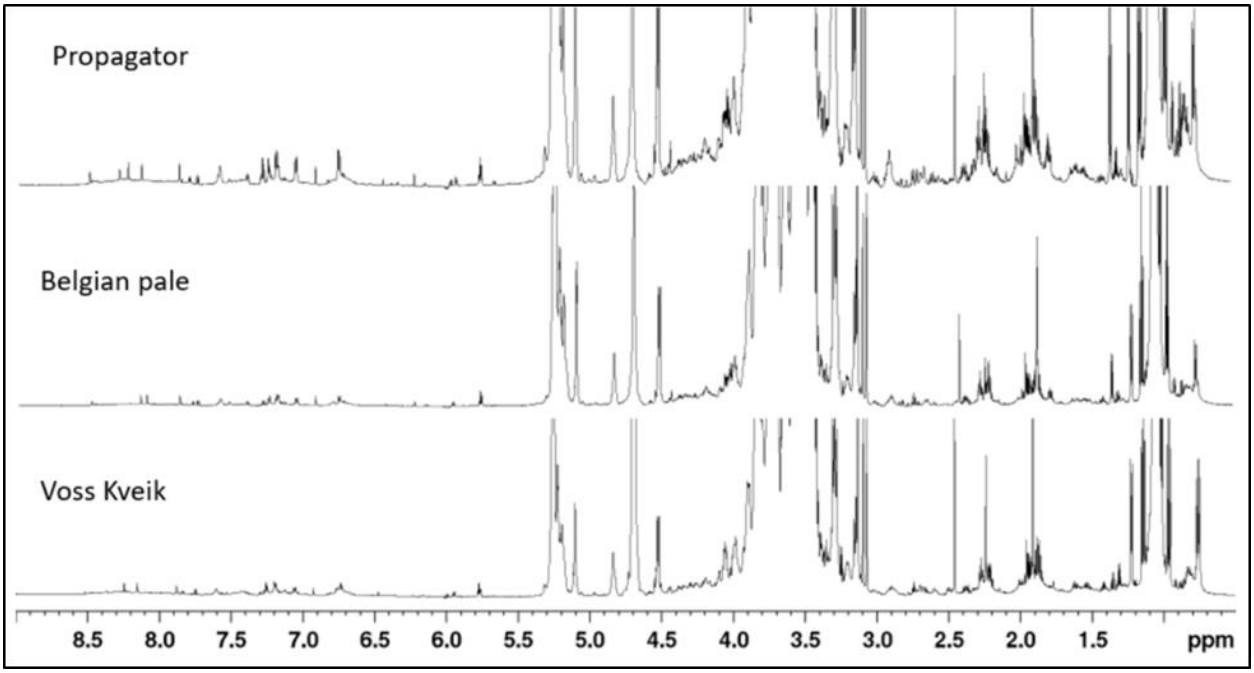

Figure 1: Comparison of beer 1H NMR spectra obtained from three different varieties of beer purchased commercially

Solvent Suppression

The excitation sculpting method proved to be superior to NOESY in terms of water suppression; however, the loss of key carbohydrate signals for the beer sample made this technique dissatisfactory for the applications in this analysis. The standard WET sequence does not work well because of the proton exchanges between water and ethanol. Here we used a modified sequence based on the noesy experiment with presaturation combined with shaped pulses. **Figure 2** compares the standard noesy presaturation experiment with the modified sequence. As can be seen, efficient suppression of all three solvent signals was achieved, thus allowing us to acquire spectra with less ADC issues (or overflow). In contrast, the standard pulse sequence is only able to efficiently suppress the water signal at δ 4.7 ppm.

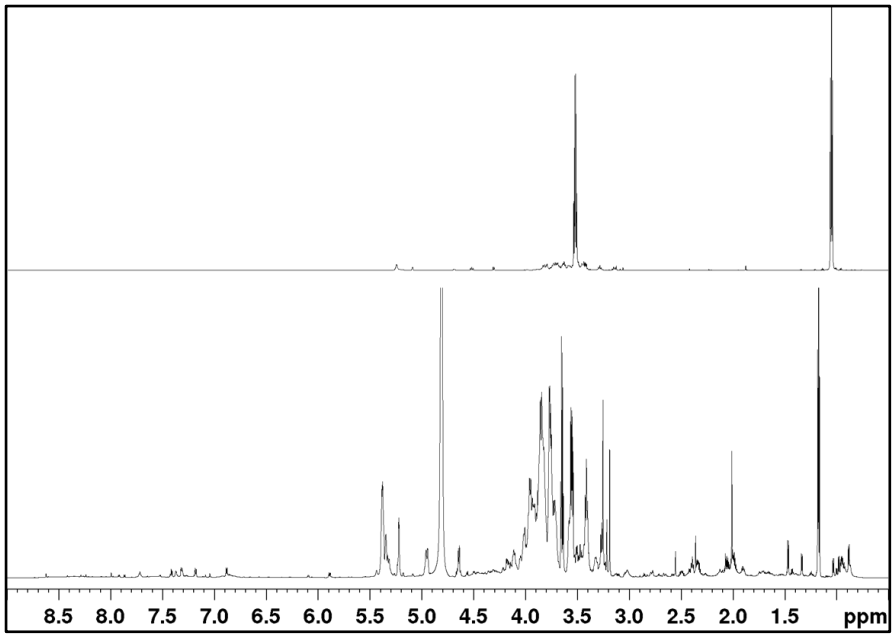

Figure 2. Comparison between the standard noesypr solvent suppression technique with the modified method used in this study.

As a next step, we applied this method for the analysis of lager and ale beer samples. The method proved to be effective even under high throughput conditions involving full automation. **Figure 3** displays the overlapped spectra obtained from the analysis of some samples used in this study.

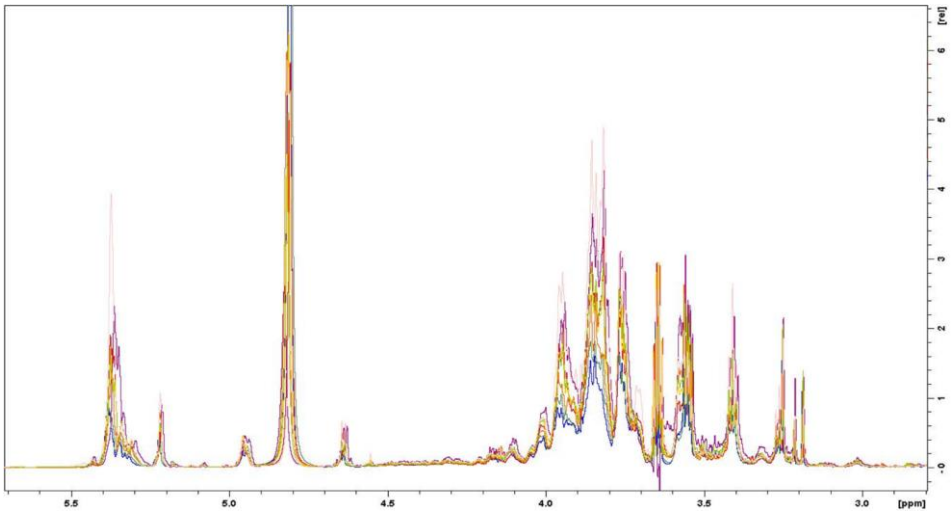

Figure 3. Overlapped spectra of some lager and ale beers analyzed in this study.

Chemometrics

Chemometric analysis was applied to data obtained by 1H NMR spectroscopy. The PCA plot obtained using the global NMR data is shown in **Figure 4** and reveals that there are not outliers among samples. In addition, a trend between sample groups was not observed. Similar results were obtained when using only the signals of carbohydrates. This is contrast to previous studies, indicating that other factors may also play a role on the chemical composition.

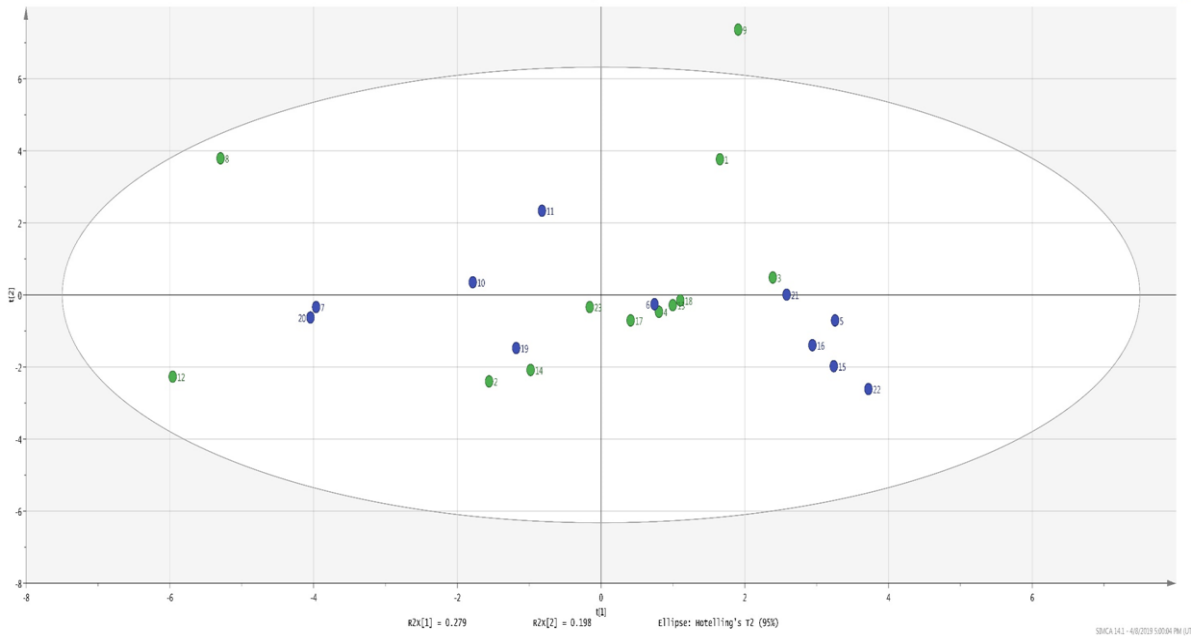

Figure 4. PCA plot generated by the 1H NMR data on 23 samples of lager (green) and ale (Blue) type.

NMR analysis of enzymatic starch hydrolysis products

NMR allows the determination of various products resulting from the starch hydrolysis using alpha-amylase alone versus alpha-amylase paired with an exogenous enzyme product including amyloglucosidase, as shown in **Figure 5A** and **5B** respectively.

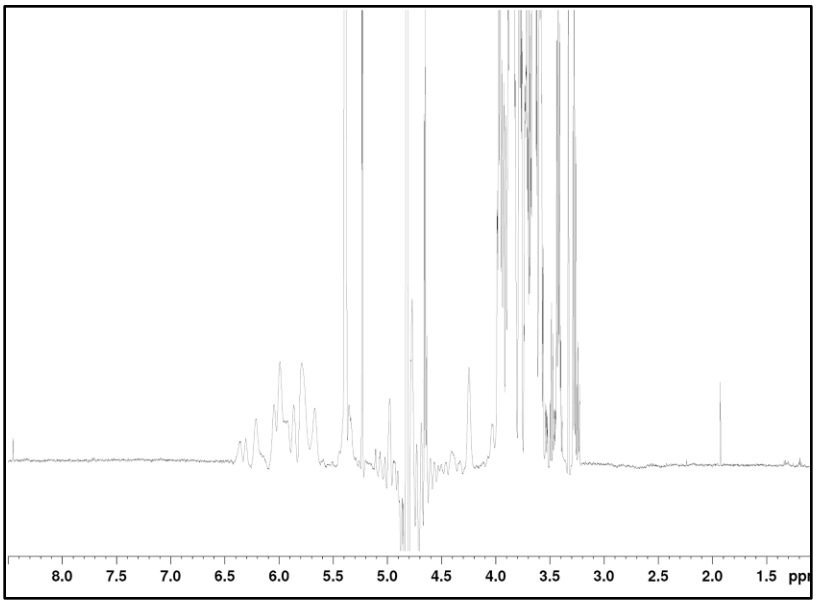

Figure 5A: Partial hydrolysis of wheat starch with *alpha-amylase*

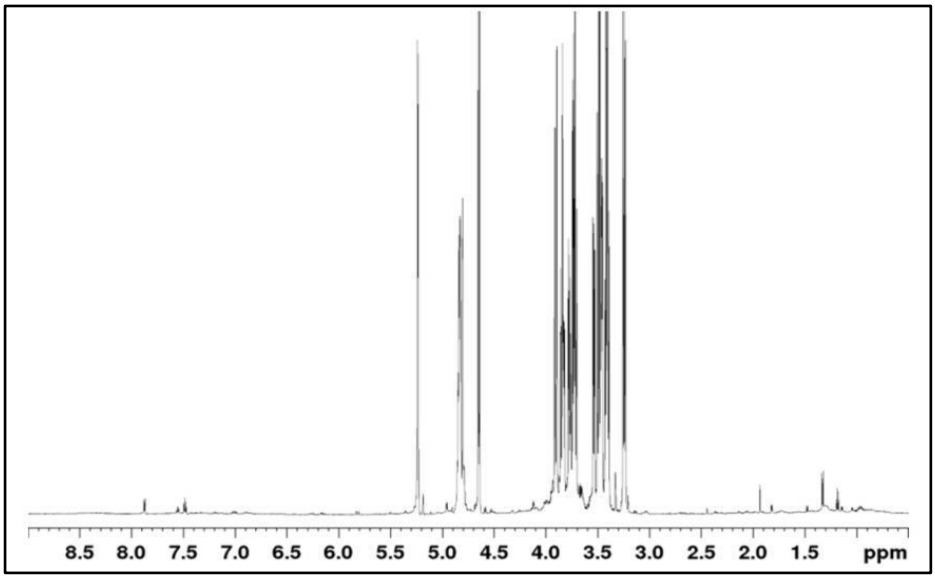

Figure 5B: Total hydrolysis of wheat starch with Diazyme glucoamylase (amyloglucosidase)

Partial vs. Total Starch Hydrolysis

There was difficulty in achieving some of the original research goals involving a kinetic study of starch hydrolysis due to an issue with the denaturation of the enzyme in a satisfactory

amount of time to limit the completion of the *alpha-amylase* hydrolysis pathway. The original hypothesis was that CD_3OD could be utilized as a simultaneous method of enzyme denaturation and as a lock solvent for the successive NMR analyses. However, there appeared to be no differences of note between the hydrolysis products at t=10 minutes versus those obtained at t=60 minutes. Small amounts of deuterated methanol provided adequate lock signal for the successive NMR analysis and barring financial limitations works well as a solvent for NMR purposes. However, its effectiveness as a tool for amylolytic enzyme deactivation requires further investigation. It is possible that this was due to enzyme concentration rather than inefficiency of methanol as an alcohol-based denaturant. Although this may arise from the non-optimized enzyme:substrate ratios and not because the method is not working properly, further studies investigating the effect of CD_3OD with and without utilizing a heat-assisted denaturation of the amylolytic enzymes are required.

Chapter 5: Conclusions and Recommendations

The application of MVSA on the 1H NMR data indicated that discrimination could not be successfully performed between samples solely based upon yeast variety alone. However, other statistical methods such as linear discriminant analysis (LDA) and random forest may be able to discriminate and provide more efficient separation among of samples and thus allow to identify which biomarkers are characteristic to each respective yeast classification.

In the recent paper by Silva et al. they were able to obtain discrimination of lager beers using 1H NMR spectroscopy[24]. However, in contrast to our work, their samples were limited to

[24] Silva, Luis Augusto Da, Danilo Luiz Flumignan, Aristeu Gomes Tininis, Helena Redigolo Pezza, and Leonardo Pezza. "Discrimination of Brazilian Lager Beer by 1H NMR Spectroscopy Combined with Chemometrics." Food Chemistry 272 (2019): 488-93. doi:10.1016/j.foodchem.2018.08.077.

the bottom-fermented categorization. In future studies, our goal is to expand upon this work, but with an emphasis on the differences between lager and ale-type beers. Based upon the results of our research so far, additional tests must be done. The modified suppression technique used was successful in the suppression of water and ethanol peaks without the obstruction of key carbohydrate resonances. However, in the recent publication by Silva et al., no ethanol suppression was used. It is possible, therefore, that adequate separation and discrimination could be achieved through analyzing the beer samples with a standard NOESY sequence, where the peaks of ethanol will be included in the analysis. In future studies, the same samples will be run using the basic NOESY sequence alone to see if this modification is effectual in the discrimination of beers. To differentiate from work conducted by Jeong et al., however, it would be useful to develop a better method in which lager and ale beers can be discriminated with ethanol suppression applied.[25]

For the development of a model for monitoring starch hydrolysis, further studies based on diminishing enzyme concentration should be conducted to verify if perceived completion of hydrolysis did not occur due to a lack of denaturation. Further validation using a total starch assay will also be conducted and compared to the results of the NMR analysis.

Part of the future goals of our research include to correlate the spectra obtained in partial and total hydrolysis of starch with those of beer. We have observed from the overlay of current spectra that there are corresponding peaks, and the next step in this process will be to determine the correspondence between the degree of hydrolysis and residual hydrolysis products in finished beer, if such a correspondence can be reasonably observed with NMR paired with statistical analysis. One particular direction of analysis would be to assess the composition of both substrates using a two-dimensional NMR method.

[25] Jeong, 466